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MAJOR REVIEW

Spreading of *Trioza apicalis* and development of "*Candidatus* Liberibacter solanacearum" infection on carrot in the field conditions

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Abstract

Carrot cultivation in Europe is suffering from infections with "*Candidatus* Liberibacter solanacearum" (CLso), a psyllid-transmitted bacterial pathogen. In this study, field experiments were carried out in Finland to separately measure the effects of psyllid feeding damage and CLso infection on the carrot root growth and to reveal the dynamics of the spreading of CLso within the field. Most of the experiments were carried out during the summers 2016 and 2017, and a follow-up sampling was performed in 2018. Carrot psyllid (*Trioza apicalis*) flight activity was monitored and carrots were sampled at 25 points within the field. Early in the season a clear spatial correlation was found between the sampling sites showing the psyllid feeding damage, that is, leaf-curling, up to the range of 40–60 m, indicating aggregation behaviour of the psyllids. No CLso infections were detected in the first sampling, which was performed before the psyllid flight peak in both years. Later, a positive correlation between the psyllid feeding damage and the CLso titre was observed. An increase in the CLso titre occurred approximately a month after the psyllid flight peak, and this increase correlated with the accumulating effective temperature sum. In 2016, both the psyllid feeding damage and CLso infection had a significant effect on the carrot root weight. The effect of CLso titre on root weight was nonlinear, that is, it intensified rapidly at the highest bacterial titres. During the colder summer of 2017 the CLso titres did not reach high enough levels in the plants to cause substantial visible symptoms and root growth reduction. Thus, it seems that in the Nordic conditions the effect of CLso infection on carrot yield is strongly dependent on the weather conditions during the growing season.

KEYWORDS

carrot symptoms, Liberibacter transmission, precipitation, psyllid trap catches, temperature sum, variogram

1 | INTRODUCTION

Carrot psyllid (*Trioza apicalis*) is a frequently occurring pest in the carrot fields in northern Europe. These psyllids overwinter on coniferous trees, especially on Norway spruce and, in the summer, they move to the carrot seedlings to feed and to reproduce. *Trioza apicalis* feeding causes a characteristic leaf-curling symptom on carrot, which becomes visible within 2 days after the feeding started (Markkula, Laurema, & Tiittanen, 1976). Nissinen, Vanhala, Holopainen, and Tiilikkala (2007) observed another symptom, a strong leaf discolouration that started to develop approximately 1 month after exposing the carrots to feeding by carrot psyllid females. Subsequently, Nissinen, Haapalainen, Jauhiainen, Lindman, and Pirhonen (2014) showed in a greenhouse experiment that "*Candidatus* Liberibacter solanacearum" (CLso) was transmitted to carrots by carrot psyllid feeding and that the two foliar symptoms were separable: leaf-curling was caused by the carrot psyllid feeding and leaf discolouration by the infection with CLso. Markkula et al. (1976) observed that even 1 hr of *T. apicalis* feeding was enough to trigger a mild leaf-curling symptom. Therefore, the *T. apicalis* feeding can be quickly detected in the carrot fields by observing this characteristic symptom. A large variation in the sizes of the carrot psyllid populations has been observed between the different years and regions (Burckhardt & Freuler, 2000; Láska, 1974; Rygg, 1977), and even within a single field (Nissinen, Piirainen, Ojanen, & Kurppa, 2000; Tiilikkala, Ketola, & Taivalmaa, 1996). Therefore, in Finland a field monitoring system based on weekly catches of carrot psyllids in yellow sticky traps (Tiilikkala et al., 1996) has been used to support decisions about applying chemical control measures since the 1990s.

Several distinct haplotypes of CLso have been found in different geographical regions. CLso haplotype C was first found in Europe in Finland in both the carrot psyllid, *T. apicalis*, and the psyllid-damaged carrots (Munyaneza et al., 2010a, 2010b). Thereafter, haplotype C has also been found in carrots in Sweden, Norway and Germany (Munyaneza, Sengoda, Stegmark, et al., 2012; Munyaneza, Sengoda, Sundheim, & Meadow, 2012; Munyaneza et al., 2015), and in *Trioza anthrisci*, a species closely related to *T. apicalis*, in United Kingdom (Sjölund et al., 2017). However, based on multilocus sequence typing (MLST) analysis, the strains of haplotype C occurring in *T. anthrisci* and its host plant *Anthriscus sylvestris* in Finland were different from the strains occurring in carrot psyllid and carrot (Haapalainen et al., 2018). Haplotype C was also detected in several seed samples of different Apiaceae family plants (Monger & Jeffries, 2018). However, there is no carrot breeding or seed production in the Nordic countries anymore, and the seed production in Europe is concentrated in the Mediterranean area, where CLso haplotypes D and E are present (Alfaro-Fernández et al., 2012; Loiseau et al., 2014; Teresani et al., 2014; Alfaro-Fernández, Hernández-Llopis, & Font, 2017; Hajri, Loiseau, Cousseau-Suhard, Renaudin, & Gentit, 2017; Mawassi et al., 2018). Bertolini et al. (2015) concluded that CLso is a seed-borne pathogen, but when Loiseau et al. (2017) repeated the experiment, the CLso-infected seeds produced noninfected carrot seedlings. On the other hand, Antolinez, Fereres, & Moreno, (2017) showed that *Bactericera trigonica* is very effective in transmitting CLso from one carrot plant to another. Recently, two seed lotsinfected with CLso haplotype D were reported in Finland (Haapalainen, Wang, et al., 2018), which suggests that there may be a potential pathway for CLso bacteria to spread via infected seeds. However, when the carrots grown from one of these infected seed lots were tested, they were CLso negative. Moreover, haplotype D was not found in carrots in the country-wide CLso survey in 2013–2014 (Haapalainen et al., 2017), which suggests that seeds are probably not the major source of CLso infections in Finland.

Despite the above-mentioned evidence against seed transmission of CLso on carrot, the question about the possibility of infected seeds as an infection source is frequently brought up in discussions with the stakeholders. Thus, it was necessary to study the association between the carrot psyllid spread and CLso spread in a field to confirm whether these psyllids are the principal source of CLso infection. A seed transmission experiment with CLso haplotype C was also performed, because all the previous seed transmission studies on carrot were carried out with plants and seed lots infected with CLso haplotypes D and E. Moreover, there is still little knowledge on the development of the CLso-related disease symptoms in carrots in the field conditions. Asymptomatic plants with detectable bacterial titres have been found both in Finland (Haapalainen et al., 2017) and in Spain (Nissinen et al., 2019), and it is unclear why the development of the discolouration symptom varies between different years. The possible relation between the summer weather in Finland and the carrot disease symptoms was previously discussed when comparing the observations in the years 2011 and 2012 (Haapalainen et al., 2017). However, no experiments have been carried out to study the effect of environmental variables, like temperature, on the development of the CLso-associated carrot disease in the field conditions in northern Europe. Furthermore, the impacts of psyllid feeding damage and CLso infection on the carrot yield have not been separately quantified in the field conditions.

The aims of this study were to define the distribution of the psyllids and CLso infections within a carrot field, to reveal the dynamics of the development of CLso infection within the field, to determine the time lap between the appearance of the vector and the disease emergence and to examine the possibility of seed transmission of CLso haplotype C. In addition, the contribution of the psyllid feeding damage and the CLso infection in the carrot root growth reduction under different weather conditions in the field was separated by statistical methods.

2 | MATERIALS AND METHODS

The fields chosen for the experiment are in an area in Finland where the occurrence of CLso was the highest in the field survey in 2013–2014 (Haapalainen et al., 2017). It is also known that in this area the percentage of carrot psyllids (*T. apicalis*) carrying CLso bacteria is high, approximately 60% (Nissinen et al., 2014). Psyllid monitoring was performed during three consecutive summers on a

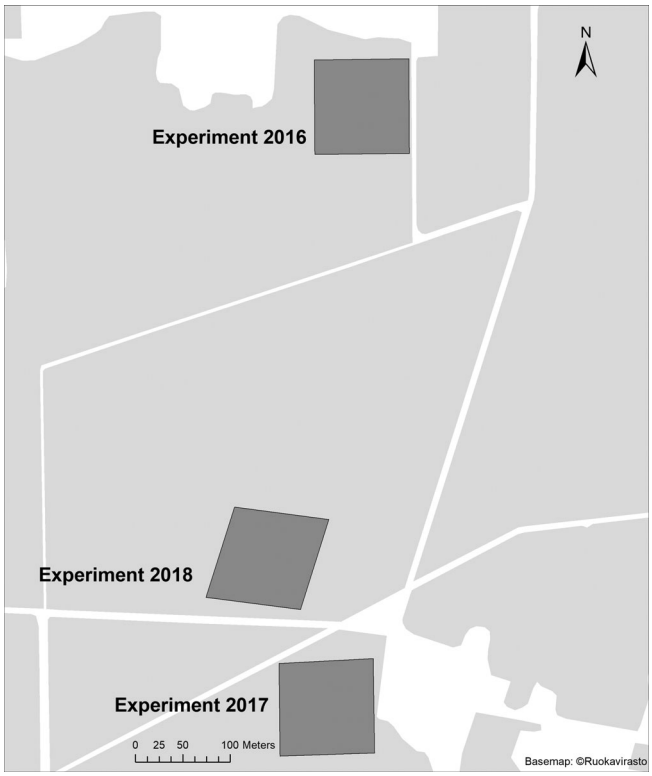

FIGURE 1 Schematic picture of the location of the experimental plots within the carrot farm in 2016, 2017 and 2018

commercial carrot farm where chemical control against psyllid infestation was used. The distance between the first and the second field plot was 530 m. The third field was just across the road to the second field, 50 m away, and the distance to the first year's plot was 350 m (Figure 1). In all the 3 years, the fields were sown with the same carrot (*Daucus carota* ssp. *sativus*) cultivar Romance. Carrot psyllid flight activity was monitored at four corners of the one-hectare experimental plot in 2016 and along the edges with eight yellow sticky traps (Catch it, Silvandersson Sweden AB, Knäred, Sweden) in 2017 and in 2018 (Figures S1–S3, Supporting Information). The traps were changed once a week from the beginning of June to the end of July, because *T. apicalis* is a univoltine species and its major flight activity takes place in these 2 months, even at different latitudes (Láska, 1974; Rygg, 1977; Tiilikkala et al., 1996). The captured carrot psyllids were identified and counted understereomicroscope.

2.1 | Sampling scheme

The plant sampling scheme was designed to study both spatial and temporal variation of the psyllid feeding damage and CLso infection. At first, experimental variograms were calculated based on the previous measurements on the distribution of carrot psyllid feeding damage in commercial fields in the years 2005 and 2006 to estimate the magnitude of the spatial variation that could occur within the field. The experimental field plot (100 × 100 m) was divided into 25 cells of 20 × 20 m in size, where the sampling point could be located in nine positions. The sampling points were situated at different distances from each other, which enabled more accurate calculation of spatial dependence compared to the design with equidistant sampling points. The small differences between sampling points are the most important when modelling the spatial dependence. Therefore, the spatial design used resulted in several sampling points within a small distance (<5 or 5–15 m) (Figures S1–S3). The same fixed sampling points were used throughout the growing season, to improve the accuracy of the temporal model. Both the sampling points and the sticky traps were located in the field with GNSS handheld-device (Trimble GeoExplorer 6000, Trimble Inc., Sunnyvale, CA).

2.2 | Plant material

Once a month during the growing season from late June to late September, carrots were sampled at each of the 25 sampling sites that had been marked in the field plot shortly after the plot was sown (Figures S1–S3). Each time, three sequential carrot plants in the same row were entirely taken as samples, resulting in 300 plant samples in total. The total number of leaves per plant and the number of damaged and discoloured leaves were recorded and the plants were weighed. Thereafter, samples for CLso analysis were cut and stored at −20°C until DNA extraction. In 2018, only one sampling was performed in September, and the samples for PCR were pressed onto Flinders Technology Associates (FTA) cards instead of cutting pieces for DNA extraction.

2.3 | Seed transmission experiment

To study the possibility of seed transmission of CLso haplotype C, first year carrot plants showing carrot psyllid feeding damage and CLso-associated discolouration symptoms in the foliage were lifted in the end of September 2017 from the same field where the experimental plot was situated. Of these naturally infected plants 50 were chosen for the seed transmission experiment. The very high titres of CLso haplotype C that can be found in the symptomatic naturally infected plants are rarely achieved in inoculation experiments performed with a limited amount of seedlings and psyllids in a greenhouse. A successful inoculation of a carrot seedling by *T. apicalis* requires a high CLso titre in the psyllid (Nissinen et al., 2014) and because of the severe feeding damage caused by *T. apicalis*, no more than three psyllids can be placed on a small seedling. So, to obtain enough infected plant material for the experiment, the plants were collected from the field. The foliage was removed from these carrots, and pieces of the petioles were cut for DNA extraction and subsequent real-time PCR for measuring the CLso titre. The roots were embedded in moist natural peat and stored in a cold storage (4–6°C) for 6 months. Thereafter, the roots were individually planted in fertilised peat sand mixture (Kylvöseos, Kekkilä Oy, Vantaa, Finland) in 3 L pots and moved into a greenhouse compartment. Three weeks after planting, three of the

new leaves developed were taken from each plant to prepare samples for the second CLso test. The flowers developed in these carrot plants in May–June were hand-pollinated with a brush. The ripe inflorescences with matured seeds were collected in July and stored in paper bags at room temperature for 6 months. The seeds were manually cleaned and counted and then cold-treated at 4–6°C for 5 weeks before sowing. Seeds were obtained from 16 different plants, and samples of 10 seeds per plant were taken for DNA extraction and subsequent CLso test by real-time PCR. Eleven seeds from each plant, altogether 176 seeds, were sown in 2 L pots in fertilised peat sand mixture (Kylvöseos, Kekkilä Oy, Vantaa, Finland) and grown in greenhouse unit at 20/15°C day/night and 20:4 hr light:dark, in similar conditions which were previously used for carrots to maintain psyllids (Nissinen et al., 2007). The carrots were grown for 2 months before samples were cut for PCR analysis and stored frozen at −20°C.

2.4 | DNA extraction and PCR analyses

Frozen 0.1 g samples of the plant leaves, cut from the petioles just above the root neck, were homogenised using FastPrep with lysing matrix A (MP Biomedicals, Irvine, CA). DNA was extracted using DNeasy Plant Mini kit (Qiagen, Hilden, Germany) and eluted in 100 µl of distilled water. Real-time PCR was performed in 20 µl reaction volume with Maxima Probe qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 5 µl of sample DNA diluted 1:100, using primers LsoF/HLBr with probe HLBp (Li, Hartung, & Levy, 2006; Li et al., 2009) for CLso and primers COXf/COXr with probe COXp (Li et al., 2006) for the plant reference gene, in separate reactions, each primer at 240 nM and probe at 120 nM concentration. The PCR program, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min, was run on LightCycler®480 (Roche). The relative titre of CLso was calculated by the Pfaffl method (Pfaffl, 2001), using experimental E values 1.90 for CLso 16S and 1.92 for plant *COX1* gene.

In 2018, the CLso infection rate was only checked at the last sampling time in September, using FTA card method. The carrot root top was cut off and the surface was pressed onto a sample area of a Whatman FTA™ MicroCard (GE Healthcare, Waukesha, WI) for 30 s. Sample discs from the carrot root prints on FTA cards were taken using a Whatman Uni-Core™ 2.00 mm punch (GE Healthcare) and placed onto a 96-well plate (Thermo Fisher Scientific). The discs were washed once with 200 µl of FTA purification reagent (GE Healthcare) and 200 µl of TE-buffer. After washing, the discs were used directly for testing CLso by end point PCR with primers OA2 (GCGCTTATTTTTAATAGGAGCGGCA; Liefting et al., 2009) and Lsc2 (GCCTCACGACTTCGCAACCCAT; Haapalainen et al., 2017) in 50 µl reaction volume, which contained 500 nM of each primer, 0.2 nM of each dNTP, Phire Hot Start II DNA Polymerase and the reaction buffer according to the manufacturer's instructions (Thermo Fisher Scientific).

For the seed transmission test, 0.1 g tissue samples were cut from the mother plant carrot petioles and DNA was extracted by the CTAB extraction protocol as previously described (Nissinen et al., 2014), with the modification that 2% polyvinylpyrrolidone 40 was added to the extraction buffer. The samples of carrot seeds were homogenised by grinding in liquid nitrogen and the DNA extraction was performed according to the CTAB protocol. Each DNA sample was dissolved in 100 µl of nuclease-free water. Of the next generation carrots, grown from the seeds produced, DNA was extracted by DNeasy Plant Mini kit (Qiagen) as described above. To test the DNA samples for CLso, the samples from the petioles of the seed-producing carrots and the seeds were tested by real-time PCR and the next generation carrots were tested by end point PCR with primers OA2 and Lsc2.

2.5 | Statistical analyses

Variogram was used to measure how much two samples taken from the field vary in the foliar psyllid feeding damage and in bacterial titre depending on the distance between those samples. First, a semivariogram $g(h)$ was calculated as

$$g(h) = \frac{1}{2N(h)} \sum_{d_{ij}=h} (y_i - y_j)^2,$$

where y_i and y_j are observations from points i and j, respectively. d_{ij} is the distance between these points and $N(h)$ is the number of points separated by the same distance h (Brooker, 2001). Typically, closer sampling points have less variability, resulting in a small value of $g(h)$. Variation increases when distance between the sampling points increases. Spatial dependence disappears at the distance where the variogram reaches its maximum. This maximum value, called sill, varied between measurement times in the current study. Therefore, relative $g(h)$ was calculated as $g(h)$/sill*100%. Analysis was performed separately for both sampling years using SAS/VARIOGRAM procedure.

Temporal variation was measured by calculating correlation coefficient from the observations at different sampling times. Analysis was performed separately for the two sampling years, using SAS/CORR procedure.

Importance of the spatial and temporal variation was estimated using a random effect model (variance component model). Point (25 points) and time (four measurement times) was included to the model as categorical random effects. The estimated variances of the spatial, temporal and unexplained variances were calculated and variances were expressed as a percentage of the sum of the three components. Analysis was performed separately for the two sampling years, using SAS/MIXED procedure.

3 | RESULTS

3.1 | Psyllid pressure

In 2016, the carrot psyllid flight started in the beginning of June and ended in mid-July, which is the usual timing in southern Finland

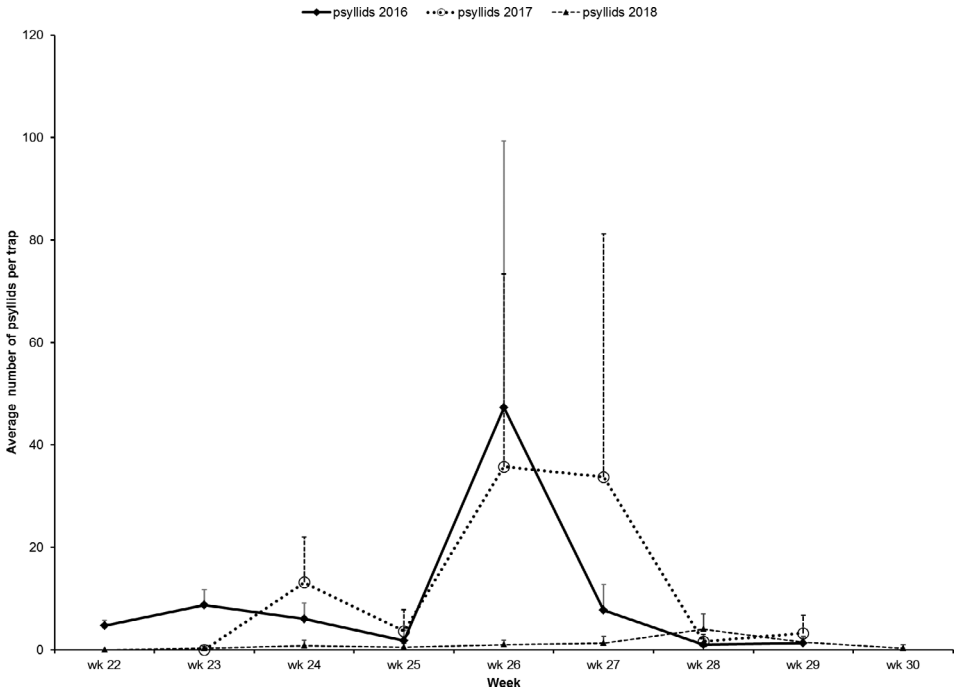

FIGURE 2 Number of carrot psyllids (*Trioza apicalis*) in yellow sticky traps in the summers 2016, 2017 and 2018. The numbers shown are the means of catches in four traps (2016) or eight traps (2017 and 2018). The error bars indicate the standard deviation

(Nissinen et al., 2007; Tiilikkala et al., 1996). The flight peaked in the last week of June (Figure 2). During the flight, the variation of psyllid counts within the observed field plot was large: the highest weekly trap catch of carrot psyllids was 123 and the lowest was four specimens in the last week of June. Especially, the high catches in the trap placed in the southeastern corner of the field suggest that the carrot psyllids were mainly entering the field from that direction (Figure S1). In 2017, the carrot psyllid flight started in the second week of June, and some flight activity was still observed in the last week of July. Thus, the flight occurred slightly later than normally in southern Finland. The differences in the trap catches between the traps placed at different locations at the edges of the field plot was substantial during the flight peak and suggested that the psyllids entered the field mainly from the northeastern side (Figure S2). The highest number of carrot psyllids per week was 147, while the lowest number was one, in the second week of July. In 2018, the carrot psyllid flight activity was low compared to the previous years. The highest weekly trap catch of carrot psyllids was 8, in the second week of July, in the traps located at the southern edge of the field plot (Figure S3). Thus, the carrot psyllid flight activity showed a large variation between the different years within the same farm and even within the same field.

3.2 | Psyllid feeding damage

Despite the chemical control measures applied at the fields, psyllid feeding damage was observed in 75, 57 and 29% of the carrots in September in 2016, 2017 and 2018, respectively. The damage percentages reflect the differences in the psyllid pressure in different years as well as the different timing of the flight peak. As the psyllid flight activity was at a substantially lower level in 2018 than in the previous years, a lower percentage of damaged plants was expected. At the last sampling time the mean leaf damage percent was 10.0% in 2016 and 5.4% in 2017 (Figures S1–S3).

To analyse the spatial variation of psyllid feeding damage in the carrot leaves within the field at each sampling time, variogram method was used to determine the autocorrelation between the sampling sites. This correlation indicates how much two samples taken from the field differ in the foliar psyllid feeding damage depending on the distance between those samples. The variogram nugget effect represents the small-scale spatial variations and indicates how "noisy" the spatial structure is. When significant spatial variation exists, the nugget effect is clearly smaller than the variation between the far-away sites. In this study the spatial autocorrelation had a small nugget effect at the first two sampling times, and significant differences in the feeding damage were observed between the different sites within the field. In 2016, a clear spatial correlation was found up to the range of 40–50 m in the beginning of the season (Figure 3a). The range is the distance beyond which the spatial autocorrelation disappears, that is, the correlation between observations is zero and variogram reaches its maximum. The correlation started to be weaker towards the end of the season and when the range exceeded 50 m. The spatial distribution revealed that the psyllid feeding damage had an aggregated structure, indicated by the small nugget size in the beginning of the season and disappearance of the autocorrelation at the range of 40–60 m. The more scattered spatial pattern of the damage in the end of the season, at the last sampling time, explains the weak spatial dependence in the short range (5–20 m). A clear temporal correlation was found in the psyllid feeding damage (r = 0.44–0.54) between all the sampling times. Temporal variation explained 16% and spatial variation 19% of the total variation in the psyllid feeding damage.

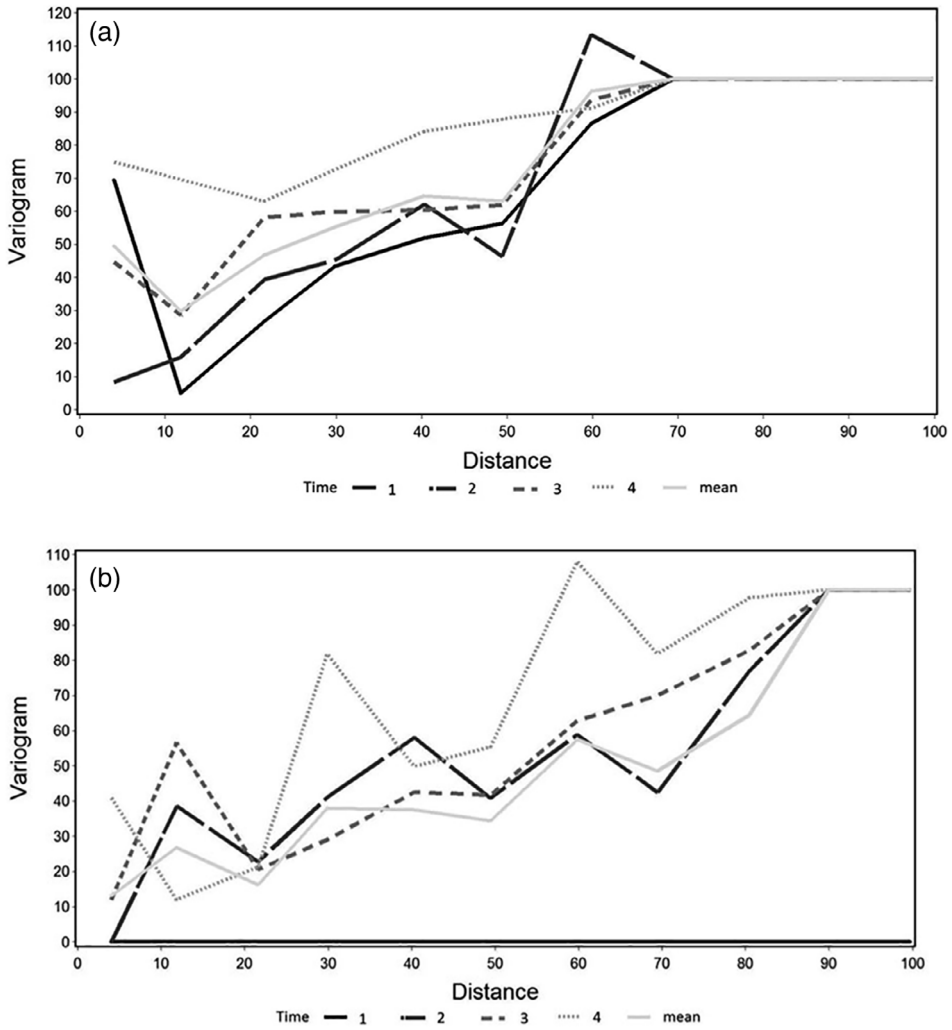

FIGURE 3 Spatial dependence of carrot psyllid (*Trioza apicalis*) feeding damage (leaf-curling) and sampling times in 2016 and 2017. The variograms for psyllid feeding damage (a) in 2016 and (b) in 2017 are shown in a relative scale, where 100% means that the observations are spatially independent

In 2017, a clear spatial correlation was found between the sampling sites where psyllid feeding damage was observed, up to the range of 60–70 m by the end of the season (Figure 3b). At the first sampling date, no psyllid damage was observed yet, which is in accordance with the trap catches showing that the flight peak was reached just before the second sampling date. A clear temporal correlation was also found in the psyllid feeding damage (r = 0 .11–0.41) between the last three sampling times. Temporal variation explained 10% and spatial variation 18% of the total variation in the psyllid feeding damage. Variograms were not calculated in 2018, because only the last sampling in September was conducted.

3.3 | Occurrence and development of CLso infection

In 2016, a clear spatial correlation was found between the sampling sites with CLso infections up to the range of 40–50 m, similarly to the psyllid feeding damage (Figure 4a). Temporal variation explained 45% and spatial variation 7% of the total variation found in the CLso titre. In 2017, a clear spatial correlation was found up to the range of 60–70 m, similarly as for the psyllid feeding damage in the leaves (Figure 4b). Temporal variation explained 29% and spatial variation 8% of the total variation found in the bacterial titre. A clear correlation was found between the psyllid feeding damage and CLso titre in the plants at the third and fourth sampling times both in 2016, r = 0.52 (p < .01) and r = 0.34 (p < .01), and in 2017, r = 0.23 (p < .001) and r = 0.44 (p < .001), respectively.

At the last sampling time in September 2016, 2017 and 2018 CLso infection was observed in 79, 52 and 7% of the carrots, respectively (Figure 5a). No CLso-infected plants were found in 2016 and 2017 at the first sampling time that was before the carrot psyllid flight peak. For CLso infections a clear temporal correlation (r = 0.41) in 2016 and a slight temporal correlation (r = 0.23) in 2017 was only found between the last two sampling times. The relative titres of CLso in the infected plants correlated with the accumulation of the effective temperature sum (Figure 5b). In 2016, the average of the relative bacterial titres detected at the second sampling time (in July) was almost four times as high as the corresponding average in 2017. In August 2016, when the temperature sum had reached 1,158°, the CLso titres were already at high levels and an increase in the number of discoloured leaves was observed. Between the samplings in July and August in both years the CLso titres increased by the order of three magnitudes, and in 2017 the relative increase was 37% higher than in 2016. Still, the final

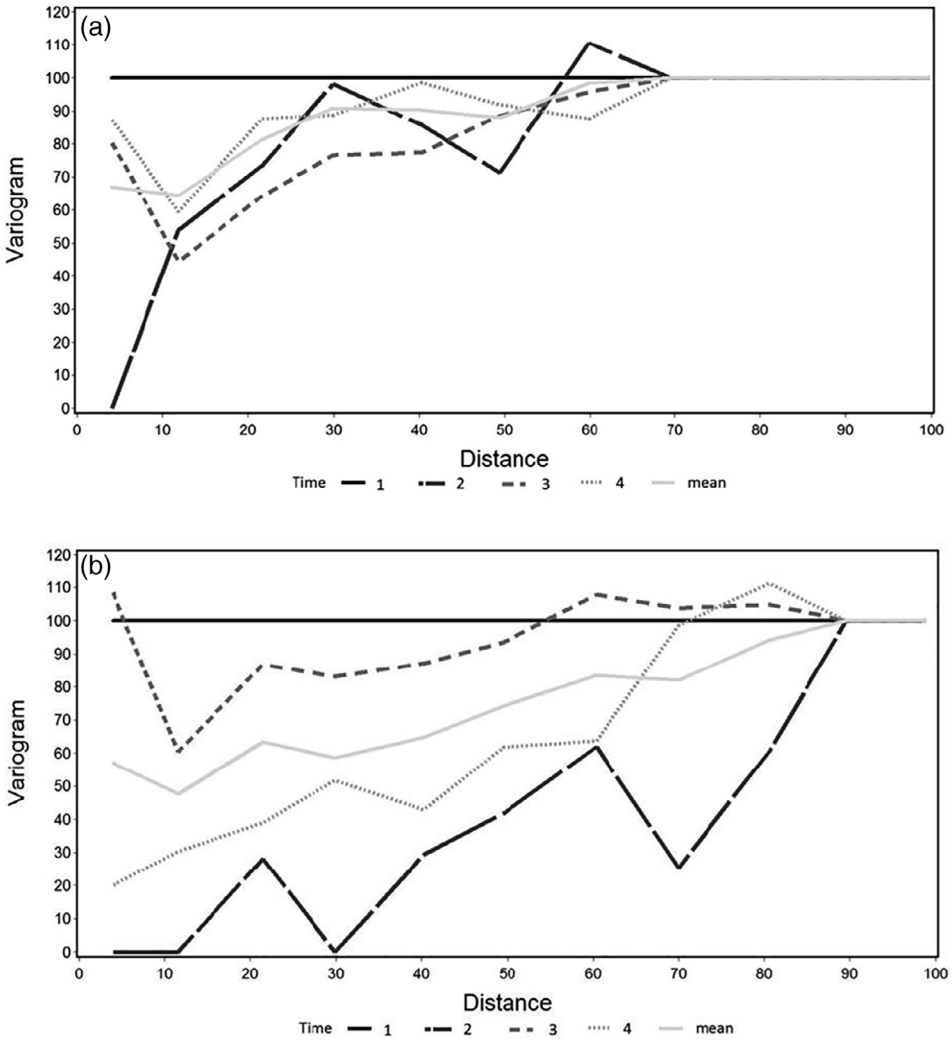

FIGURE 4 Spatial dependence of the relative titre of "*Candidatus* Liberibacter solanacearum" (CLso) within a field (a) in 2016 and (b) in 2017. The variograms are shown in a relative scale, where 100% means that the observations are spatially independent

titres detected in September were lower in 2017 than in the previous year. Hardly any discoloured leaves were observed in the carrots in 2017 (Figure 5b). These analyses were not performed in 2018, because only the last sampling in September was conducted.

3.4 | Carrot root weight

In 2017, both the leaves and the main root of the seedlings were more advanced at the first sampling time than in 2016, despite the lower effective temperature sum. Moreover, the increase in the root weight was more rapid between the first and the second sampling time in 2017 than in 2016 (Figure 5c). Based on the coefficient of determination, the number of true leaves was the best predictor for carrot root weight during all the 3 years. In 2016, adding psyllid feeding damage rate and CLso titre to the model increased the coefficient of determination which was 23, 64, 52 and 36% at the first, second, third and fourth sampling time, respectively. At the first sampling time, psyllid feeding damage rate and CLso titre explained 15% of the variation, at the second time only 1%, at the third time 17%, and at the fourth time 19%. The effect of psyllid feeding damage was significant at the first, third and fourth sampling times ($p < .01$) and the effect of CLso titre was marginally significant at the fourth sampling time ($p = .09$). In 2017, the coefficients of determination for the model were 27, 28, 42 and 55% at the first, second, third and fourth sampling time, respectively. However, adding the psyllid feeding damage rate and CLso titre to the model, in addition to the number of true leaves, did not increase the coefficient of determination. In 2018, the coefficient of determination for the polynomial true leaf model for the root weight was $r = 0.72$ and the significance $p = .01$.

3.5 | Seed transmission

All the 50 symptomatic carrots collected from the field in the autumn 2017 were CLso positive, and after 6 months of storage the CLso bacteria in the roots were still viable and able to move up and infect the new emerging leaves. The average CLso titre in the old first year leaf petioles was 6.9×10^5 and in the new second year leaf petioles 3.9×10^5, with large variation, standard deviations 5.6×10^5 and 5.8×10^5, respectively. The seed samples from all those 16 plants that

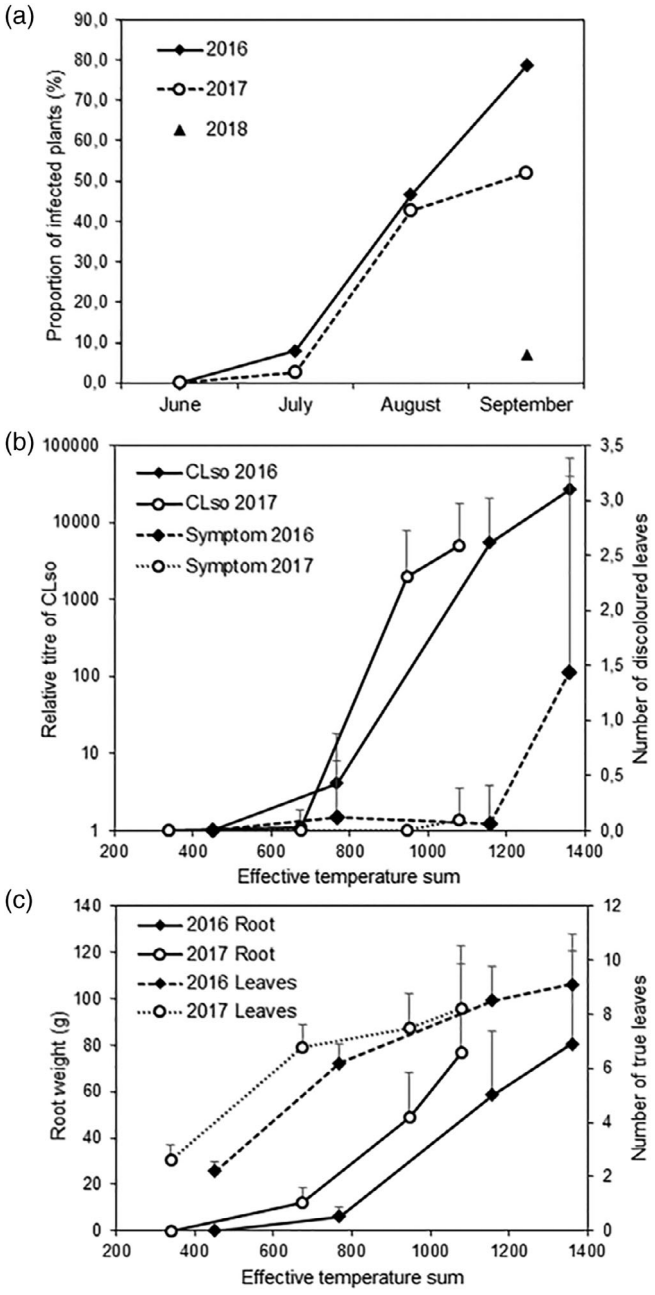

FIGURE 5 Development of "*Candidatus* Liberibacter solanacearum" (CLso) infection and the related symptom in carrots during the summers 2016 and 2017. (a) The proportion of infected plants at different sampling times, (b) the average relative titres of CLso in the infected plants and the average number of discoloured leaves in the plants (in the second *y*-axis) in relation to the effective temperature sum. (c) Development of the carrot plants during the growing seasons 2016 and 2017. The average root weight and the average number of true leaves (in the second *y*-axis) are shown in relation to the effective temperature sum at different sampling times

produced an adequate amount of mature seeds tested positive for CLso (Table 1). However, none of the next generation carrots grown from these 16 infected seed lots were positive for CLso, when they were tested after growing for 2 months in a greenhouse.

4 | DISCUSSION

Carrot growth, the amount of carrot psyllid feeding damage and the course of CLso infection in the carrots were studied in the field conditions during two very different summers in 2016 and 2017. The statistical analysis revealed that carrot root development was best predicted by the number of true leaves. In 2016, the psyllid feeding also had a significant effect on the root weight, whereas the CLso titre only showed a marginally significant effect at the last sampling time. In 2016, the carrot growth was restricted by the low precipitation during the psyllid flight peak, keeping the carrots at growth stages more susceptible to the psyllid feeding. The higher effective temperature sum, in comparison to the summer 2017, clearly contributed to the higher relative titre of CLso as well as the development of discolouration symptom in the plants. In the cooler and more rainy summer in 2017 neither the psyllid feeding damage nor the bacterial titre had a significant effect on the carrot root weight. However, the quality of the carrot roots was not assessed in this study; in a previous study the carrots exposed to psyllid feeding were found bitter and tougher than the nonexposed controls (Seljåsen et al., 2013). Our observations suggest that environmental conditions substantially affect the damage caused by both the carrot psyllid and CLso, thus confirming the previous suppositions on the role of weather conditions (Haapalainen et al., 2017). In addition, Nissinen, Pihlava, Latvala, and Jauhiainen (2020) addressed the effect of environmental stresses on the success of psyllid control: both cold and rainy and hot and dry summers were suboptimal for the control programs. The analysis of the spatial distribution of the psyllid feeding damage within the field revealed that it had an aggregated structure. A clear correlation was found between the psyllid feeding damage and the CLso titre in the plants at the third and fourth sampling times in both years, confirming *T. apicalis* as the main source of CLso infections on carrot.

In all the 3 years 2016–2018, the carrot psyllid flight started when the carrots were still small seedlings (cotyledon to one-leaf stage), and thus vulnerable to the feeding damage (Nissinen et al., 2007, 2012). However, in 2016 the psyllid flight peaked more than a week earlier than in 2017, which posed a higher risk of psyllid feeding damage on the carrot seedlings. On the other hand, the more vigorous growth of the seedlings at the early stage in 2017 may explain the lower feeding damage percent observed at the last sampling time in 2017. Nissinen et al. (2007) showed that leaf-curling level of 10–15% led to root growth reduction in greenhouse, which is in line with the field observations in this study and Nissinen et al. (2020).

In both 2016 and 2017, the highest trap catches at the flight peak exceeded 100 specimen per trap, indicating a high pressure of potential vectors of CLso. These trap catches at the flight peak were higher than those observed by Tiilikkala et al. (1996), but at the same level as observed previously during the 2000s (Nissinen et al., 2007). The trap catches did not, however, reach the record levels of 2014, which was approximately 500 individuals per trap per week (Haapalainen et al., 2018). In 2018 the psyllid pressure was much lower than in the two previous years, which resulted in a lower level

TABLE 1 Relative titre of "*Candidatus* Liberibacter solanacearum" (CLso) haplotype C in the petioles and seeds of the symptomatic carrots used as mother plants, the viability of the seeds and the infection status of the seedlings

Mother plant ID	CLso titre in the first year leaf petioles	CLso titre in the second year leaf petioles	CLso titre in the seed sample	Proportion of seeds germinated (%)	Proportion of CLso positive seedlings (%)
11	31,148	64,506	16,494	55	0
15	191,377	101,719	46,461	100	0
19	631,337	1,240,472	83,327	18	0
21	180,335	8,408	57,545	21	0
22	283,402	33,291	95,320	91	0
28	45,800	1,060	119,095	18	0
29	29,128	284,504	208,623	73	0
36	756,197	48,774	30,230	18	0
37	474,828	5,910	23,546	73	0
38	60,221	22,487	9,421	73	0
39	602,136	12,749	75,040	82	0
41	1,174,812	4,885	72,848	45	0
44	676,603	767,780	48,402	27	0
46	1,457,716	480,193	14,208	64	0
47	2,271,634	28,525	44,295	36	0
50	110,150	8,706	73,206	18	0

of psyllid feeding damage as well as a lower number of CLso infected plants. A clear temporal correlation was found in the psyllid feeding damage in 2016 between all the sampling times and in 2017 between the last three sampling times (no damage observed at the first sampling). This may be explained by the fact that the carrot psyllid flight reached its peak between the first and the second sampling time in both years, and most of the leaf damage developed during that time. The leaf-curling caused by the adult carrot psyllid feeding persists in those leaves in which it has once formed. In spatial variation the nugget effect was small in the beginning of the season and the psyllid feeding damage was more similar in the plants within small distance from each other, which suggests aggregation behaviour of the psyllids. In the end of the season the spatial pattern of the feeding damage was more scattered. The nugget value was higher in the last sampling times, which may reflect differences in psyllid pressure or in the success of psyllid control at different sites. In some plants the psyllids might have been removed by the insecticides immediately after landing, preventing them from reproducing. In the other plants more psyllids per plant have landed or the psyllids managed to stay alive longer and lay eggs, and later the nymphs developed. The psyllid nymphs cause less severe symptoms than the adults (Markkula et al., 1976) and they are less mobile than the adults, and thus the nymphs mainly strengthen the symptoms in the same plants where the adults have been feeding. The range of the spatial correlation of the psyllid feeding damage was found to be 40–50 and 60–70 m in 2016 and in 2017, respectively. In practice, this means that in small fields the damage can be assumed to be the same throughout the field plot as is observed at the edges, whereas in the bigger field plots of several hectares area the damage observations are independent after the 50–60 m distance. Therefore, there may be large differences in both the psyllid feeding damage and the psyllid flight intensity within the field, as observed in this study and previously reported by Nissinen et al. (2000) and Tiilikkala et al. (1996).

The relative titre of CLso significantly correlated with carrot psyllid feeding damage in both years 2016 and 2017, suggesting that carrot psyllids had transmitted CLso to the plants later found CLso-positive. The intensity of the psyllid flight also had a clear effect on the CLso infection rate. In 2018 the trap catches of *T. apicalis* at the flight peak were only 8–11% of the previous year's catches, and accordingly CLso infection was only detected in 7% of the carrot samples in September 2018. Further support to the hypothesis that CLso is mainly vector-borne is given by the result that none of the seedlings grown from the highly infected seed lots in a greenhouse was CLso positive, suggesting that seed transmission of CLso haplotype C is a rare event if happening at all. This result is in accordance with the study by Loiseau et al. (2017), in which they concluded that for CLso haplotype D seed is not a major pathway of transmission. The CLso detected in the infected carrot seeds may be confined to the seed coat and not able to enter the developing embryo. Altogether, our observations support the conclusion that CLso haplotype C is not seed-borne but is spread by the psyllid *T. apicalis*.

The increase in CLso titre in the field-grown carrots that was observed between the late July and late September followed similar pattern as previously observed in the psyllid exposure experiments in a greenhouse. Nissinen et al. (2012) observed the development of 6.0–8.3 discoloured leaves on average within 2.5 months after the psyllid exposure. Furthermore, Nissinen et al. (2014) showed that the number of discoloured leaves positively correlated with the high bacterial titre in the plants. In line with these results, a correlation was

found between the incidence of the discolouration symptom and CLso infection in a field survey (Haapalainen et al., 2017). In the plants exposed to carrot psyllids in greenhouse, the discolouration symptom was observed on average at the effective temperature sum of 1,133.6°C, calculated on the base temperature of 5°C (Nissinen et al., 2007). In the field conditions, the effective temperature sum in late September was 1,361°C in 2016 and 1,079°C in 2017. The average temperatures in June and July 2017 were from 1–2 °C lower than the long-time average and 1.9° lower than in 2016 (Table S1). The lower temperatures might have restricted the multiplication of CLso, first inside the psyllids in June and then in the infected plants in July, which then resulted in the lower CLso titres observed. In August 2017, the temperature was closer to the long-time average, but the precipitation was higher than usual. The cloudy and moist weather could have prevented the development of strong foliar symptoms in the infected carrots. It is possible that the level of photosynthesis is not too much impaired by the CLso infection until the leaf discolouration symptom appears, as previously observed with other pathogens like luteoviruses in beet root (Rossing, van Oijen, van der Werf, Bastiaans, & Rabbinge, 1992). This could explain the result in this study that the effect of CLso titre on the carrot root weight was not significant in 2017, whereas it was marginally significant in 2016, when the high CLso titre was accompanied by visible discolouration symptoms in the field.

Munyaneza, Sengoda, Buchman, and Fisher (2012) observed that the development of CLso infection in potato was reduced at temperatures between 12 and 17°C. In this study, the mean temperatures in the field varied between 10.4 and 14.9°C in the summer 2017, while in 2016 the range of mean temperatures was from 11.6 to 16.8. As CLso infections in carrot still developed in these conditions, it is possible that the temperature range of CLso haplotype C is lower than that of the haplotypes A and B infecting Solanaceous plants in America. However, further studies are needed to define the range of optimum temperatures for CLso haplotype C.

In 2016, the effect of carrot psyllid feeding damage on the carrot root weight was significant at three sampling times, while the effect of CLso titre was marginally significant (p = .09) at the last sampling time only. The number of true leaves was found to be the best predictor for the root weight. In 2016, the carrots were on average at 2.6-leaf stage at the first sampling time, when the carrot psyllid flight had already started. Precipitation in June 2016 was lower than in 2017, which resulted in slower development of new leaves and smaller increase in root weight in the beginning of the season. In contrast, the lower effective temperature sum did not seem to restrict the early carrot growth in 2017. The poorer growth of the carrot seedlings in 2016 may have contributed to the significantly worse psyllid feeding damage than in 2017. These field observations are in line with the previous results (Nissinen et al., 2007, 2012), which showed that in greenhouse experiments the carrots were most vulnerable to psyllid feeding at the cotyledon to two-leaf stage and acquired some tolerance at the four-leaf stage. Our findings in this study suggest that early psyllid feeding is a more significant factor affecting the root development than the CLso bacterial titre. These findings are in line with Nissinen et al. (2020), who found that the carrot psyllid feeding damage in the leaves was negatively correlated with the weight of the carrot root. Previously, Nissinen et al. (2014) observed that some of the carrots with strong foliar discolouration had such a high CLso titre that the phloem vessels were filled with the bacteria and many sieve cells had collapsed. This kind of damage in the phloem vessels is likely to lead to reduction in the sugar transport to the roots and thus restrict the root growth. Altogether, our findings suggest that at the early stages of carrot growth the psyllid feeding is a more significant factor affecting the root development than the CLso infection. The effect of the CLso titre becomes significant only in the end of the season, close to the harvest time.

The first symptoms of zebra chip disease on potato were observed in the 1990s in America, although there is evidence that CLso has coexisted with the potato psyllid *Bactericera cockerelli* for a much longer time (Zeilinger et al., 2017). Zeilinger et al. (2017) have also shown that *B. cockerelli* population abundance in relation to other Hemiptera species has increased during the last century. However, further work is needed to link this to the zebra chip outbreaks. Similarly, the recent findings by Monger and Jeffries (2018) in the historical seed collections of Apiaceae have shown that CLso has occurred in the Apiaceae cultivation system in the European-Mediterranean region already for decades. However, the first epidemics of discolouration symptoms on carrot leaves were not observed before 2007 in Finland and in Spain. Casteel, Hansen, Walling, and Paine (2012) consider CLso an endosymbiont-like bacterium, as it seems to regulate the host plant defence response to benefit the obligate psyllid host's success on the host plant. Thus, CLso might have belonged to the bacterial flora of psyllids for a very long time. However, the global warming may affect these plant–vector–pathogen interactions. The first decade of the 21st century was the warmest since the measurements were started in the 19th century (Coumou, Robinson, & Rahmstorf, 2013). Milder winters in northern Europe may have enhanced the growth of the populations of the vectors like *T. apicalis*, for which the trap catches have been dramatically higher in the 2010s than previously (Haapalainen, Latvala, et al., 2018; Tiilikkala et al., 1996). At the same time, higher effective temperature sums during the growing seasons may have enhanced the multiplication of the CLso bacteria in the crop plants, leading to more frequent occurrence of symptoms. In this study, we observed considerably higher bacterial titres and more discolouration symptom in the CLso-infected carrots at the end of the season 2016 than in 2017, and the increase in the bacterial titre clearly correlated with the accumulation of the effective temperature sum during both years. Thus, we hypothesize that the global warming has changed the environmental conditions for the benefit of both the insect vector species and the CLso bacteria, leading to the recent disease outbreaks on different crops.

5 | CONCLUSIONS

In this study we found that following the course of CLso infection in the field conditions during two different growing seasons revealed the importance of the environmental conditions, especially the two

factors, amount of precipitation and effective temperature sum. These two environmental factors had opposing effects on both the psyllid feeding damage and CLso infection. The results suggest that a rapid development of carrots at the early growth stages protects them against the psyllid feeding damage. Although the beginning of the growing season in May–June has become drier during the last decades, however, the carrots are seldom irrigated in spite of the drought. In the light of our results, it may be profitable to irrigate the carrots at early growth stages to enhance the growth in order to protect the carrots against the psyllid feeding damage. The analysis of spatial distribution within the field revealed that psyllid feeding damage had an aggregated structure. Therefore, there may be large within-field differences in the amount of psyllids and the psyllid feeding damage. On the other hand, the effect of CLso titre on the root weight was observed to be nonlinear, that is, the effect was strongest with the highest bacterial titres. The increase in CLso titre seemed to be temperature-dependent and the highest increase started at the end of July, which is the warmest month in Finland. Therefore, with the rising temperatures, the yield reduction in carrots as a result of CLso infection is expected to occur with higher frequency in the future climate. In practice, the early sown carrots, which are harvested for the fresh markets, are most probably the ones which can escape both the psyllid feeding damage and CLso, because they have already passed the cotyledon to two-leaf stage before the carrot psyllid flight starts. In contrast, the late sown storage carrots will probably suffer from both the psyllid feeding and CLso infection.

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